Angiogenesis in liver fibrosis

Adlia, Amirah

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CELL-SPECIFIC DELIVERY OF INTERFERON ALPHA AND ITS ANTI-ANGIOGENIC ACTIVITY IN HEPG2 CELLS

AMIRAH ADLIA, CATHARINA REKER–SMIT, VALENTINA FRANCIA, GENY M.M. GROOTHUIS, KLAAS POELSTRA

DIVISION OF PHARMACOKINETICS, TOXICOLOGY AND TARGETING, DEPARTMENT OF PHARMACY, UNIVERSITY OF GRONINGEN, THE NETHERLANDS

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ABSTRACT

It is well known that the hepatic stellate cells, differentiated into myofibroblasts, play a major role in fibrogenesis. However, although hepatocytes make up ca. 80% of the liver mass, their role in liver fibrosis has remained under-examined. Hepatocytes produce important angiogenic factors and since it is known that angiogenesis emerges in parallel with liver fibrosis, it can be hypothesized that hepatocytes play a role in fibrogenesis by regulation of angiogenesis. Based on this hypothesis, drugs aimed to influence angiogenesis in hepatocytes could be developed as a potential new antifibrotic therapy. In order to unravel the role of hepatocytes in angiogenesis in liver fibrosis, we aimed to interfere with the angiogenic balance regulated by hepatocytes through delivery of interferon alpha (IFNα), a cytokine with anti-angiogenic properties, to the hepatocytes. To specifically deliver IFNα to the hepatocytes, IFNα was conjugated to galactose-polyethylene glycol (galactose-PEG) in order to increase the hepatocyte delivery by binding to the asialoglycoprotein receptors (ASGPR) that are expressed abundantly on hepatocytes. This galactose-PEG-IFNα (GPI) showed comparable biological activity with respect to STAT1 phosphorylation as native IFNα in HepG2 cells. The binding of GPI to the ASGPR on HepG2 cells was confirmed by competition with the ASGPR ligand lactosylated human serum albumin (LacHSA). The STAT1 phosphorylation of GPI but not of free IFNα was partly inhibited by LacHSA. Both GPI and free IFNα induced a decrease in vascular endothelial growth factor (VEGF) secretion and increase in thrombospondin-1 (THBS-1) protein expression in hepatocytes. In addition, the tubule formation of human umbilical vein endothelial cells (HUVEC) *in vitro* was inhibited by incubating the HUVEC in conditioned medium of HepG2 cells treated with GPI or IFNα but not by that of control HepG2. In conclusion, both IFNα and its galactose-PEG conjugate GPI induce an anti-angiogenic effect in HepG2 cells. In the future, the effect of GPI on fibrogenesis will be studied *in vivo* in a mouse model of fibrosis.

1. INTRODUCTION

Liver fibrogenesis is mainly sustained by hepatic myofibroblasts (MF) which represent a heterogeneous population of cells [1-4]. Hepatic stellate cells (HSC) are non-parenchymal cells in the liver known to be the leading actor in liver fibrogenesis, because hepatic MF are mainly originated from activated HSC [1,2]. On the other hand, the role of hepatocytes, which comprise ca. 80% of the liver volume, in liver fibrogenesis has not been completely elucidated. Hepatocytes have been proposed to contribute in liver fibrosis through a process of epithelial to mesenchymal transition, but this issue remains controversial [1,5,6]. In addition, hepatocytes in normal and cirrhotic livers express several important angiogenic factors, such as vascular endothelial growth factor (VEGF) and thrombospondin-1 (THBS-1) [7-9], suggesting a role in angiogenesis.

Angiogenesis emerges in parallel with liver fibrogenesis [9-12]. However, it has not been understood yet whether angiogenesis occurs as a consequence of fibrosis or whether it has a causal role in fibrosis [13,14]. Since hepatocytes produce important angiogenic factors, we hypothesized that hepatocytes play role in liver fibrosis through supporting angiogenesis. Thus, we aimed to explore the role of hepatocytes in angiogenesis in liver fibrosis.

In order to investigate the contribution of hepatocyte by interfering with their role in angiogenesis, we exposed HepG2 cells to interferon alpha (IFNα). IFNα has been used in the treatment of hepatitis C and several cancers [15]. Besides its antiviral activity, IFNα is also known to have an anti-angiogenic effect due to its capability in downregulating the expression of the pro-angiogenic factors VEGF in many human cancer cells and inhibits endothelial cell migration *in vitro* and *in vivo* [16-19]. However, interferon alpha receptors (IFNAR) are expressed in most of the tissues in the body [20] and IFNα treatment is known to cause many unfavorable side effects, which hamper the use of IFNα as anti-fibrotic drug and to study the role of hepatocytes in angiogenesis. In order to solve this, we designed a construct by coupling galactose-polyethylene glycol (galactose-PEG) to IFNα. This galactose-PEG-IFNα (GPI) will be recognized by the asialoglycoprotein receptors (ASGPR) which are expressed abundantly on the hepatocytes [21,22], thereby reducing the exposure of other cells in the body. The galactose part was pre-coupled with PEG to increase the biological half-life of IFNα. In this study, we present the characterization of the GPI and its biological activity on angiogenesis in HepG2 cells *in vitro*.

2. MATERIALS AND METHODS

2.1 Synthesis and characterization of GPI

2.6 nmol of human recombinant interferon alpha 2b in PBS (19.4 kDa, Jena Biosciences, Jena, Germany) was coupled to 130 nmol of galactose-polyethyelene glycol-succinimidyl carboxymethyl

(galactose-PEG-SCM) ester (5 kDa, Jenkem Technology, Beijing, China). The reaction was carried out for 1 h at room temperature and continued overnight at 4 °C with vigorous shaking. Purification was done by extensive dialyzing against PBS with Slide-A-Lyzer™ with 20K molecular weight cut-off (Thermo Fisher Scientific, Rockford, USA). The construct (GPI) was characterized by western blot analysis and MALDI-TOF.

For western blot analysis, the GPI construct and the free IFNα as a positive control were applied on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and after electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h in tris-buffered saline with 0.1 % Tween 20 (TBST) containing 5% non-fat dried milk and was further incubated with anti-interferon alpha 2b mouse monoclonal antibody (1:200; Abcam, Cambridge, UK) for overnight at 4 °C. The membrane was then washed with TBST and incubated for 2 h at room temperature with a secondary horseradish peroxidase (HRP)-coupled anti-mouse IgG antibody (DAKO). After washing three times with tris-buffered saline (TBS), the protein bands on the membrane were visualized using electrochemiluminescence (ECL) detection reagent.

The molecular weight of GPI was determined with MALDI-TOF Voyager DE™-Pro (Applied Biosystems, Foster City, USA). GPI (1 mg/mL) and standard (1 μM bovine serum albumin) were spotted onto a MALDI-plate well and allowed to dry. Next, matrix solution (sinapinic acid in 0.1% trifluoroacetic acid and 50% acetonitrile) was overlaid on the sample and allowed to dry. The instrument was operated with an accelerating voltage of 25 kV and was set to acquire mass spectral peaks with mass/charge (m/z) ratios from 10 to 80 kDa.

2.2 *In vitro* experiments

2.2.1 Cell lines

HepG2 cells were obtained from ATCC (Manassas, USA) and human umbilical vein endothelial cells (HUVEC) were obtained from pooled donors (Lonza, Walkersville, USA). HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad) supplemented with 10% fetal bovine serum (FBS) and antibiotics (50U/mL penicillin and 50 ng/mL streptomycin). HUVEC were cultured in Endothelial Basal Medium-2 (EBM-2) supplemented with EGM-2-MV bullet kit (Lonza, Walkersville, USA) containing VEGF, IGF-1, EGF, bFGF, hydrocortisone, ascorbic acid, heparin and 2% FBS.

2.2.2 STAT1 phosphorylation

HepG2 cells were incubated with 10 and 100 ng/mL GPI or IFNα for 4 and 24 h. The concentration of GPI was calculated based on the concentration of IFNα, which was determined

with an ND-1000 spectrophotometer at 280 nm (Fisher Scientific, Landsmeer, the Netherlands). The influence of PEG on the IFNα quantification was tested by adding unconjugated gal-PEG to IFNα which did not result in a change in absorbance.

STAT1 phosphorylation was measured by western blot analysis using an antibody specific for phosphorylated STAT1 (pSTAT1). The cells were lysed in loading buffer (0.5 mM Tris-HCl pH 6.8, Glycerol, 8% SDS, 400 mM dithiothreitol and 0.0125% bromophenol blue) on ice, sonicated for 5 sec and boiled at 90 °C for 5 min. The cell lysate from each sample was applied on the 10% SDS-PAGE gel and after electrophoresis, transferred to a PVDF membrane. The membrane was blocked for 1 h in TBST containing 5% non-fat dried milk and was further incubated with anti-pSTAT1 (Tyr701) rabbit monoclonal antibody (1:1000; Cell Signaling Technology, Danvers, USA) and anti-GAPDH mouse monoclonal antibody (1:10000, SIGMA, Missouri, USA) overnight at 4 °C. The membrane was then washed with TBST and incubated for 2 h at room temperature with a secondary HRP-coupled anti-rabbit IgG antibody (DAKO) for pSTAT1 and HRP-coupled anti-mouse IgG antibody (DAKO) for GAPDH. After washing three times with TBS, the protein bands on the membrane were visualized using the VISIGLO™ HRP Chemiluminescent Substrate Kit (Amresco, Solon, USA).

2.2.3 Inhibition with Lactosylated HSA (LacHSA)

LacHSA was prepared according to the method described by van der Sluijs *et al.* [23]. This LacHSA contained ca. 25 galactose moieties per molecule of HSA. HepG2 cells were first incubated with 1.3 or 13 nM of LacHSA for 10 min. Thereafter, 10 ng/mL of IFNα or GPI were added and the cells were incubated for 4 h. After 4 h, the cells were lysed and STAT1 phosphorylation was analyzed with western blot according to the method described in 2.2.2.

2.2.4 The effect of GPI on VEGF secretion and THBS-1 expression by HepG2 cells

HepG2 cells were incubated with 10 and 100 ng/mL IFNα and GPI for 24h. The incubation medium was collected and the cells were lysed in loading buffer (composition is described in method 2.2.2). The amount of VEGF in the collected incubation medium was analyzed by using the Human VEGF-A ELISA reagent kit (Thermo Fisher Scientific, Rockford, USA).

Western blot analysis of the cell lysates was performed using anti-THBS-1 mouse monoclonal antibody (1:200; Thermo Fisher Scientific, Rockford, USA) and anti-GAPDH mouse monoclonal antibody (1:10000, SIGMA, Missouri, USA) overnight at 4 °C. The membrane was then washed with TBST and incubated for 2 h at room temperature with a secondary temperature with a secondary HRP-coupled anti-mouse IgG antibody (DAKO). After three wash steps with TBS, the

protein bands on the membrane were visualized using the VISIGLO™ HRP Chemiluminescent Substrate Kit (Amresco, Solon, USA).

2.2.5 *In vitro* angiogenesis: endothelial cell tube formation assay

The effect of IFNα and GPI on the production of VEGF by the HepG2 cells was tested *in vitro* using HUVEC according to the protocol described by Arnaoutova *et al.* [24]. HepG2 cells were incubated with medium alone or medium containing 10 and 100 ng/mL IFNα or GPI for 24 h. The medium was collected and stored at -80 °C until further analysis.

HUVEC were cultured in the EBM-2 medium and split when the confluence was around 80% until they reached passage 3. The cell suspension ($4x10^4$ cells/well) was plated in a 96-well plate coated with Matrigel® (Corning, USA). HepG2 conditioned medium was transferred to the HUVEC and incubated for 4 h. The tube formation of the HUVEC was observed by microscopy and pictures were taken at 40x magnification. The length of the tubes was measured with ImageJ by using Angiogenesis Analyzer Plugin.

2.3 Statistics

Data are presented as mean ± standard error mean (SEM). The statistical analysis was performed by using one-way ANOVA and followed with Dunnett's Multiple Comparisons. A p-value < 0.05 was considered significant.

3. RESULTS

3.1 Characterization of Gal-PEG-IFNα (GPI)

The successful coupling of IFNα with activated galactosylated-PEG-SCM (5 kDa) was confirmed by western blot using an anti-IFNα antibody **(Fig. 1A)** and by MALDI-TOF **(Fig. 1B)**. **Fig. 1A** shows two bands of free IFNα (lane 2 and 3) representing the monomer and the dimer of this protein. GPI showed several bands (lane 4) of coupling products and the presence of a minor amount of free IFNα. The percentage of free IFNα in the GPI sample was calculated by quantification of the bands of the monomer IFNα in the samples of free IFNα and GPI using GeneTools software (SynGene) and showed that less than 10% unconjugated IFNα (monomer) remained present without any unconjugated dimer. However, it is not possible to assess the apparent molecular weight (MW) of the GPI constructs because it is known that pegylation influences the mobility in the gels [25]. Therefore, to analyze the MW of GPI and to determine the amount of gal-PEG coupled to IFNα, MALDI-TOF analysis was applied. The result can be observed in **Fig. 1B** showing that the GPI construct consists of a mixture of 1, 2, and 3 Gal-PEG

coupled to IFNα. The MALDI-TOF spectrum was used to identify the molecular weight of the GPI and not the relative abundance of each of the constructs, as the area under the peak does not reflect the abundance of the molecule but rather the ionazibility and/or the ion stability [26]. Since PEG is known to suppress the ionization of the protein [25,27], the intensity of the various GPI constructs in the MALDI-TOF analysis is dependent on the degree of pegylation.

Based on both the western blot and the MALDI-TOF results, we conclude that the majority of the construct consists of IFNα conjugated with 1 galactosylated-PEG, and that constructs with 2 and 3 galactosylated PEG molecules are present in a lower amount.

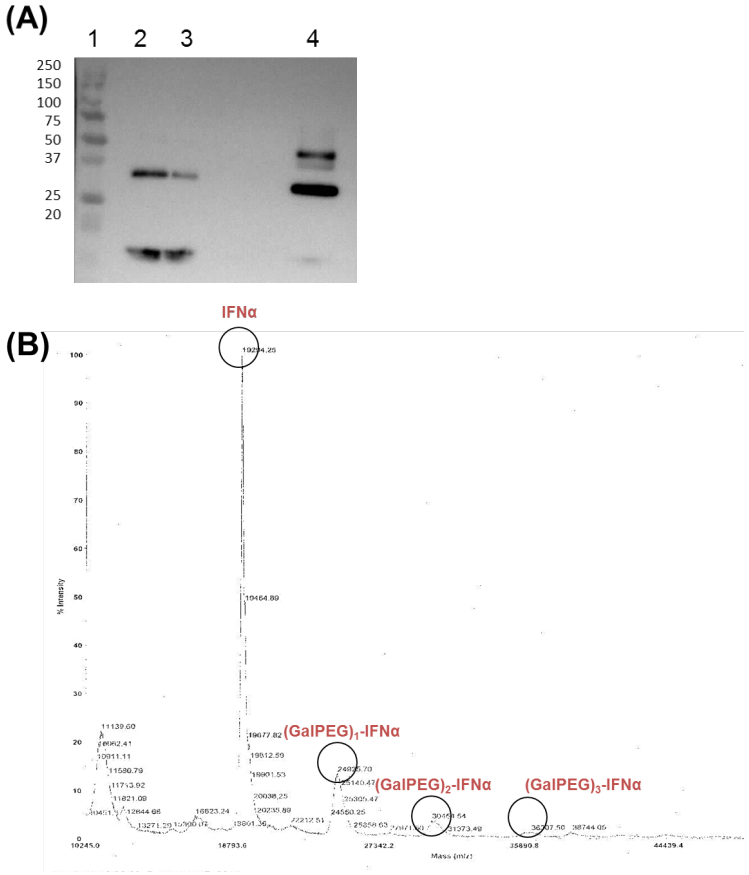


Fig. 1 Characterization of GPI. **(A)** Western blot analysis using anti-IFNα antibody; lane 1: marker; lane 2: IFNα 50 ng/mL, lane 3: IFNα 25 ng/mL, and lane 4: GPI 50 ng/mL. **(B)** MALDI-TOF analysis of GPI showing the free IFNα and three different coupling products of galactose-PEG - IFNα.

3.2 STAT1 phosphorylation

STAT1 phosphorylation to pSTAT1 is an essential step in the response to IFNα. The results in **Fig. 2** showed that after 4 h, both free IFNα and GPI induced phosphorylation of STAT1. After 24h, the amount of pSTAT1 in HepG2 cells treated with either IFNα or GPI was strongly diminished.

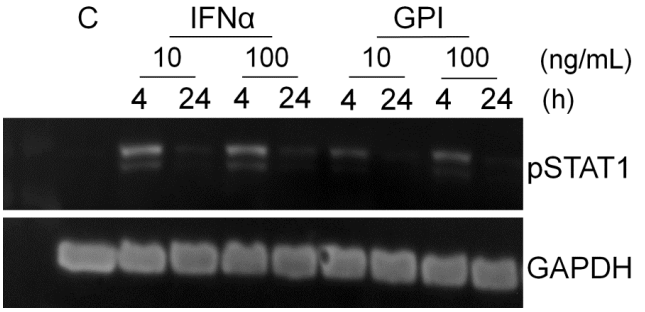


Fig. 2 Western blot of phosphorylated STAT1 (pSTAT1) in HepG2 cells treated with IFNα and GPI and GAPDH expression as loading control. HepG2 cells were incubated for 24 h without any treatment (C); or treated with 10 and 100 ng/mL of IFNα or GPI for 4 and 24h.

3.3 Inhibition with LacHSA

In order to show the potential contribution of binding of GPI to the ASGPR to the pSTAT1 phosphorylation, we did an inhibition study *in vitro* using HepG2 cells. HepG2 cells express both IFNAR and ASGPR [28,29]. Our results showed that the inhibition with 1.3 nM of LacHSA (molar ratio= 1:2.6) resulted in the reduction of pSTAT1 formation by GPI, but the pSTAT1 formation by IFNα remained unchanged **(Fig. 3A, B)**. The inhibition with a higher amount of 13 nM LacHSA (molar ratio= 1:26) did not further reduce the pSTAT1 formation **(Fig. 3A, B)**.

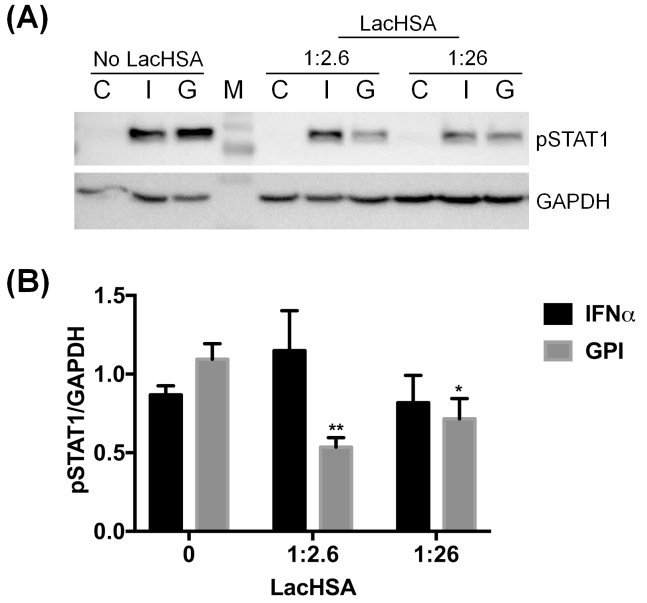


Fig. 3 (A) Effect of lactosylated HSA (LacHSA) on STAT1 phosphorylation in HepG2 cells treated with medium alone (C), 10 ng/mL of IFNα (I) or GPI (G) and co-incubated without LacHSA or with 1.3 nM or 13 nM LacHSA with molar ratio of 1:2.6 and 1:26; GAPDH was used as loading control. **(B)** Quantification of the results showing that the addition of LacHSA reduced the STAT1 phosphorylation of GPI but the activity of IFNα remained unchanged (n=5).
Data are presented as means (± SEM)
*p<0.05; **p<0.01

3.4 The effect of GPI on the expression of angiogenic factors in HepG2 cells

VEGF is a potent pro-angiogenic factor, which is secreted in the liver mostly by hepatocytes [7, 8], while THBS-1 is known as anti-angiogenic factor produced in hepatocytes [9]. The effect of GPI and IFNα on the expression of these angiogenesis factors was determined by measuring the secretion of VEGF in the HepG2 medium and the expression of THBS-1 in the cells after 6 and 24 h. After 6 h, neither IFNα nor GPI showed an effect on VEGF secretion and THBS-1 expression (data not shown). After 24 h of incubation, a small but significant reduction of VEGF secretion by the HepG2 cells treated with IFNα and GPI **(Fig. 4A**) was found. The effect was similar at both concentrations.

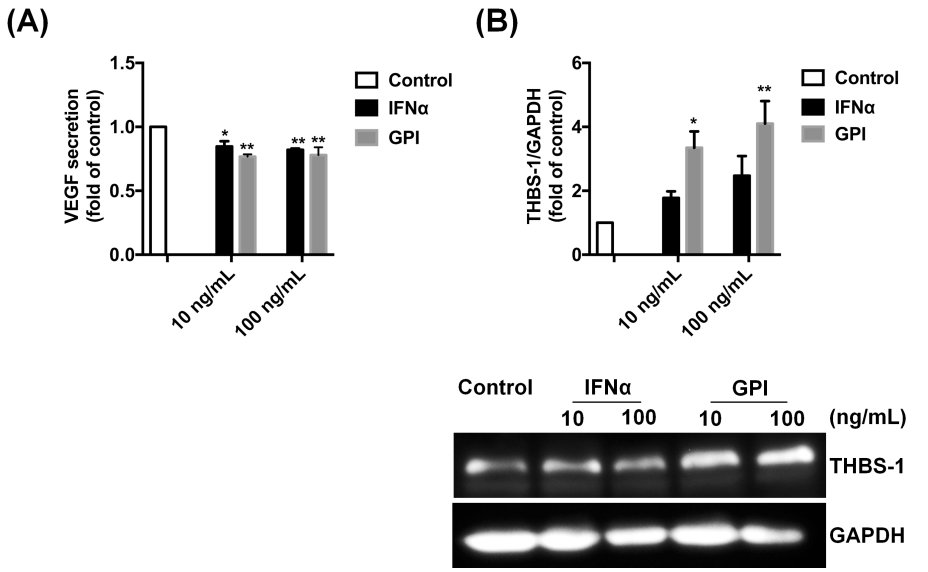


Fig. 4. The amount of the pro-angiogenic factor VEGF **(A)** and the anti-angiogenic factor THBS-1 **(B)** expressed by HepG2 cells after 24 h treatment with 10 and 100 ng/mL of IFNα or GPI expressed as fold of control (untreated cells). IFNα and GPI significantly decreased VEGF-A secretion. GPI induced a significantly higher expression of THBS-1, but the effect of IFNα was not significant.
Data are presented as means (± SEM); n=3
*p<0.05; **p<0.01; compared to control

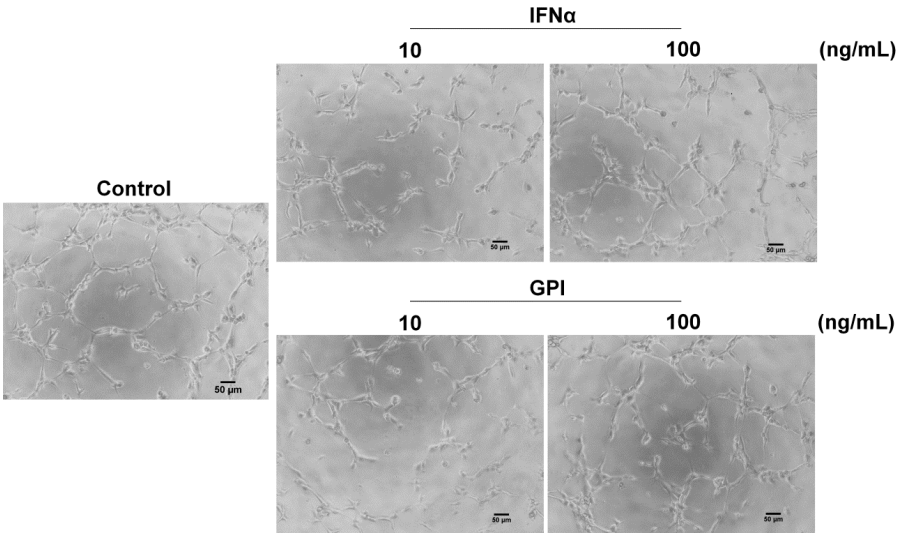
In addition, we observed a dose-dependent increase of the expression of THBS-1 in HepG2 cells treated with IFNα and GPI after 24 h of incubation **(Fig. 4B)**, which was only significant for GPI **(Fig. 4B)**.

3.5 The effect of GPI on HUVEC tube formation

In order to investigate the effect of GPI on angiogenesis in HepG2 cells, an endothelial cell tube formation assay with HUVEC was performed. The HUVEC were incubated in the medium of untreated or IFNα- or GPI-treated HepG2 cells. After 4 h incubation of HUVEC with medium of untreated HepG2 cells, the HUVEC formed a stable interconnected network of tube-like structures **(Fig 5A)**, which was not formed in unconditioned medium (results not shown). The tube length was measured as an indicator of the angiogenic response. The HUVEC that were incubated in HepG2 cells medium treated with 10 ng/mL of IFNα or GPI showed smaller tube lengths compared to untreated HepG2 cells medium **(Fig. 5A, B)**. However, no effect was observed in cells treated with conditioned medium from HepG2 cells treated with the higher concentrations of both IFNα and GPI (**Fig.5A, B**). We also tested whether the effect on HUVEC

was due to the presence of IFNα and GPI in the medium by using medium containing both compounds incubated for 24 h without cells. The result showed that there was no direct effect of IFNα or GPI on HUVEC tubule formation (data not shown).

(A)



(B)

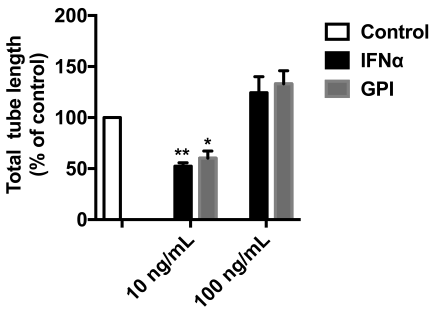


Fig. 5 (A) Microscopic images and **(B)** quantification of tube formation of HUVEC treated with HepG2 cells conditioned medium, i.e. control (untreated HepG2 cells) and HepG2 cells treated with 10 and 100 ng/mL of IFNα or GPI for 24 h, showing that incubation of HUVEC in conditioned medium from HepG2 cells treated with 10 ng/mL of IFNα or GPI resulted in shorter tube length compared to conditioned medium of untreated HepG2 cells and no effect was observed in medium of HepG2 cells treated with 100 ng/mL of IFNα or GPI.
Data are presented as means (± SEM). n=5 (1 batch of HUVEC treated with conditioned medium of 5 independent HepG2 experiments)
*$p<0.05$; **$p<0.01$; compared to control

4. DISCUSSION

In this study, we designed a modified IFNα, with 1-3 moieties of galactose-PEG coupled to IFNα, aimed to be recognized by the ASGPR of HepG2 cells, and we evaluated its biological activity *in vitro*. IFNα is known to have anti-angiogenic properties [16-19] and with this construct, we ultimately aim to deliver IFNα to the hepatocytes in the liver *in vivo* via the ASGPR. This would enable us to investigate the role of hepatocytes in the regulation of angiogenesis during fibrosis and to elucidate the role of angiogenesis in fibrosis. We used HepG2 cells as *in vitro* model to investigate the effect of GPI because these cells express both the ASGPR and IFNAR [28,29]. The results of the western blot and MALDI-TOF analysis showed that IFNα was successfully coupled to multiple moieties of galactosylated PEG, varying from mono to trivalent. These amounts are supposed to be sufficient for selective recognition by the ASGPR, which is abundantly expressed on hepatocytes[30]. Although previous studies have shown that the binding affinity increases with increasing numbers of galactose moieties [30], oligonucleotides coupled to one gal-PEG showed hepatocyte specific uptake *in vivo* in the rat, which could be inhibited by galactosylated bovine serum albumin [31]. This indicates that coupling one galactose-PEG to IFNα could be sufficient to induce internalization via the ASGPR.

The *in vitro* results showed that the coupling reaction did not influence the biological activity of GPI to a large extend, as phosphorylation of STAT1 by GPI was similar to that of free IFNα in HepG2 cells. Binding of GPI to the ASGPR was verified by the competitive inhibition with LacHSA. LacHSA has about 25 lactose molecules coupled to HSA and was previously shown to be taken up by the ASGPR [23]. Indeed, a significant decrease was observed in the STAT1 phosphorylation by GPI when this construct was added together with 1.3 nM LacHSA. This effect was not observed in cells that were treated with free IFNα, indicating that the effect of GPI was partly mediated by the ASGPR. The presence of 13 nM LacHSA did not further decrease the STAT1 phosphorylation of GPI, indicating saturation of the ASGPR at 1.3 nM. The remaining effect on the phosphorylation of STAT1 was apparently mediated by the IFNAR. As a result of uptake via the ASGPR, one would expect that GPI should have a higher effect compared to free IFNα. However, the results showed that the biological activity of both compounds were similar. This can be explained either by decreased biological activity of GPI due to the conjugation conditions or by saturation of STAT1 phosphorylation in the cells. The former option seems unlikely as IFNα exposed to the same reaction conditions as applied during conjugation, showed a similar STAT1 phosphorylation as free IFNα (data not shown). Our result showed that even with higher concentration of free IFNα or GPI, there was no further increase of STAT1

phosphorylation in the HepG2 cells, supporting the explanation that saturation of STAT1 phosphorylation is the reason for the lack of increased effect of GPI.

There are two different possibilities for the effect of GPI via the ASGPR. First, GPI can be taken up by the ASGPR and internalized via receptor-mediated endocytosis. Although in general via this route the ligands are usually degraded by the enzymes in lysosomes, in some cases it has been shown that the ligand can escape the endosome [32]. This route would result in binding of the GPI construct to the intracellular domain of the IFNAR. A construct of siRNA coupled to gal-PEG has been shown to be internalized by the ASGPR and to exert a gene silencing effect [33]. In that study, the disulfide bond between the PEG and siRNA was cleaved in the cytoplasm allowing the release of free siRNA [33]. In our study, we conjugated PEG to the IFNα via a non-cleavable amide bond to the lysine residues. Although cleavage of GPI apparently is not necessary for activation of the extracellular receptor, it remains unknown whether PEG has to be cleaved from the GPI to exert its effect after the cellular uptake [34]. Interestingly, Jung et al. showed that there was no difference in silencing activity of siRNA conjugated to PEG via either cleavable or non-cleavable linkage [35]. Another possibility of ASGPR mediation of GPI uptake is facilitating the binding of the construct to the IFNAR on the surface of the HepG2 cells, as a form of receptor cross talk as illustrated in **Fig. 6**.

The effects of IFNα and GPI on angiogenesis were tested by determining the secretion of VEGF and THBS-1 by HepG2 cells in the medium. While VEGF is known as a potent angiogenesis activator, THBS-1 is known as angiogenesis inhibitor [36]. When HepG2 cells were treated with IFNα or GPI, the VEGF secretion was significantly reduced but the decrease was relatively small. In addition, treatment with GPI significantly increased the expression of THBS-1 in a dose-dependent manner. However, the effect of IFNα on THBS-1 expression was not significant. From this result, it seems that GPI has a stronger effect on THBS-1 compared to IFNα. Based on these data, we concluded that the treatment of HepG2 cells with GPI resulted in excretion of factors that may induce an anti-angiogenic effect.

To test this anti-angiogenic effect, we evaluated the effect of conditioned medium of HepG2 cells on the formation of a tubular network by migration of endothelial cells *in vitro* using HUVEC[24]. Treatment of HUVEC with conditioned medium obtained from untreated HepG2 cells showed the migration of HUVEC and the formation of a tubular network, as a result of basal excretion of VEGF [37]. The length of the tubes was used as a parameter in this study to indicate the angiogenic response of conditioned medium of HepG2 cells treated with IFNα and GPI. The results showed that conditioned medium of HepG2 cells treated with the lower dose of IFNα and GPI significantly reduced the length of the tubes formed by migrating HUVEC, in line with the

reduced VEGF and induced THBS-1 excretion. However, the effect was not seen with medium of HepG2 cells exposed to the higher concentration of both compounds. Although this result does not match with our findings in the effect of these compounds on the expression of the angiogenic activator and inhibitor, this results can be explained by the review of Reynolds *et al.* who observed that some anti-angiogenic compounds exhibited a bell-shaped or U-shaped dose response [38], as was also found for the anti-angiogenic effect of IFNα in human bladder cancer [39].

In conclusion, we present in this study a biologically active galactose-PEG modified IFNα that is recognized by the ASGPR and induces an anti-angiogenic reaction in HepG2 cells. We hypothesize three possible ways for GPI to exert its biological activity **(Fig. 6)**, i.e. through uptake of the ASGPR, followed by escape from the endosomes and binding to the intracellular IFNAR, through binding to and activation of the IFNAR on the cell surface directly or with the help of ASGPR, where the binding to the ASGPR enables a better contact of the IFNα moiety with the IFNAR, without internalization of the ligand by the ASGPR. This construct will be further investigated *in vivo* to evaluate its effect on liver fibrogenesis.

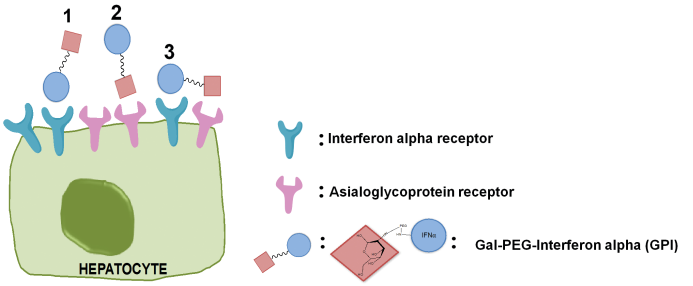


Fig. 6 Schematic representation of the hypotheses on the binding mechanism of GPI to exert its effect. 1: via interferon alpha receptor (IFNAR); 2: via the asialoglycoprotein receptor (ASGPR); and 3: via receptor cross talk of the IFNAR and the ASGPR.

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